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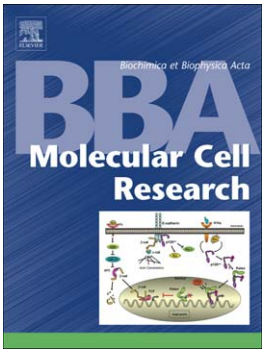

HGF/c-Met signaling promotes liver progenitor cell migration and invasion by an epithelial-mesenchymal transition-independent, phosphatidyl inositol-3 kinase-dependent pathway in an in vitro model

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"HGF/c-Met signaling promotes liver progenitor cell migration and invasion by an epithelial-mesenchymal transition-independent, phosphatidyl inositol-3 kinase-dependent pathway in an in vitro model"

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Abreviations: Epithelial-mesenchymal transition, EMT; Extracellular matrix, ECM; Golgi complex, GC; HPC, hepatic progenitor cells; hepatocarcinoma, HCC; HGF, hepatocyte growth factor; metalloproteases, MMPs; small interfering RNA, siRNA; tight junctions, TJ; tissue inhibitors of metalloproteases, TIMPs.

Abstract

Oval cells constitute an interesting hepatic cell population. They contribute to sustain liver regeneration during chronic liver damage, but in doing this they can be target of malignant conversion and become tumor-initiating cells and drive hepatocarcinogenesis. The molecular mechanisms beneath either their pro-regenerative or pro-tumorigenic potential are still poorly understood. In this study, we have investigated the role of the HGF/c-Met pathway in regulation of oval cell migratory and invasive properties. Our results show that HGF induces c-Met-dependent oval cell migration both in normal culture conditions and after in vitro wounding. HGF-triggered migration involves F-actin cytoskeleton reorganization, which is also evidenced by activation of Rac1. Furthermore, HGF causes ZO-1 translocation from cell-cell contact sites to cytoplasm and its concomitant activation by phosphorylation. However, no loss of expression of cell-cell adhesion proteins, including E-cadherin, ZO-1 and Occludin-1, is observed. Additionally, migration does not lead to cell dispersal but to a characteristic organized pattern in rows, in turn associated with Golgi compaction, providing strong evidence of a morphogenic collective migration. Besides migration, HGF increases oval cell invasion through extracellular matrix, process that requires PI3K activation and is at least partly mediated by expression and activation of metalloproteases. Altogether, our findings provide novel insights into the cellular and molecular mechanisms mediating the essential role of HGF/c-Met signaling during oval cell-mediated mouse liver regeneration.

Keywords: c-Met, liver progenitor cell, migration, invasion, epithelial-mesenchymal transition, PI3K

1. Introduction

Oval cells are bipotent adult hepatic progenitor cells (HPC) that expand after chronic severe damage, migrate and invade into liver parenchyma and differentiate into mature epithelial liver cells (hepatocytes and cholangiocytes), thus actively contributing to repopulate the liver and restore liver function. This oval cell-mediated regenerative process is often referred to as "oval cell response" or "ductular reaction" due to the fact that proliferating oval cells organize into ductular structures or ductules [1]. Their role in regeneration of the damaged liver has been convincingly proven mostly based on their presence in advanced stages of human liver diseases, surrounded by immature hepatocytes, which indicates an ongoing regeneration response; and their demonstrated capacity to repopulate the liver upon transplantation in animal models of liver injury. Hence, they have been considered as the second line of defense against liver injury [2]. Unfortunately, the repopulation process is often not efficient enough and hepatic failure can not be always prevented. Strikingly, recent evidences suggest that a sustained oval cell response may indeed aggravates fibrosis [3]. Furthermore, it is also recognized that deregulation of the oval cell activation/differentiation pathway can result in malignant transformation of these cells that subsequently become tumor-initiating cells and drive hepatocarcinogenesis [4]. It becomes clear that, in spite of their relevance in liver pathophysiology, our knowledge about the complex and heterogeneous biology of HPCs is still limited. Despite all these uncertainties, their plasticity, repopulation capacity and differentiation potential keep high expectations regarding their use as suitable sources or targets for clinical application strategies aimed at accelerating or improving regeneration for the treatment of liver diseases.

The regulation of the oval cell response seems to be as complex as the response per se. Thus, a combination of genetic studies in rodents and in vitro studies have allowed to identify a number of cytokines and growth factors, which are somehow involved in oval cell regulation [5,6]. Hepatocyte growth factor (HGF) originally identified as a strong mitogen for mature hepatocytes, has been demonstrated to be a growth factor essential not only during liver development [7], but also to promote a successful regenerative response after acute and chronic liver damage [8-10]. HGF triggers its biological activities through binding to a single tyrosine kinase receptor

named c-Met. Activation of Met by HGF induces a variety of cellular responses, including proliferation, differentiation, survival, motility and extracellular matrix (ECM) degradation [11,12]. Interestingly, all these biological activities appear to be part of a unique c-Met-activated morphogenic response known as "invasive growth", which operates during normal development and regeneration but it also has a malignant counterpart responsible for cancer progression and metastasis [13].

Previously, we have demonstrated that HGF/c-Met signaling is essential to promote oval cell survival in vitro via autocrine and paracrine mechanisms involving PI3K activation [14,15]. Consistent with its important regulatory role in oval cell function, in vivo data from c-Met mutant mouse models have shown that the absence of c-Met results in a failure of the stem/progenitor cell-mediated regenerative response and ultimately death from liver failure. Interestingly, loss of c-Met has more profound effects than just a reduction in oval cell pool, affecting multiple cellular processes required for regeneration, including oval cell differentiation and migration [16]. The effects of HGF/c-Met signaling on the regulation of cell migration are well known. Indeed, HGF was also identified as the "Scatter factor" due to its capacity to induce disassociation of cell-cell contacts and increase motility in epithelial cells, resulting in cell scattering in vitro [17]. On the other hand, HGF also induces extracellular matrix proteolysis and invasion in various types of non-tumoral and tumoral cells, including hepatoblasts and hepatocarcinoma (HCC) cell lines, activities that appear to contribute to both in vivo liver repopulation capacity and tumor invasiveness and metastasis, respectively [18,19].

Despite these enlighting observations, the regulation of the complex migratory process involved in the regenerative response mediated by oval cells has not been characterized. In this study, we have investigated the relevance of the HGF/c-Met signaling pathway on regulation of mouse oval cell migration and invasive properties and the mechanisms mediating its effects. For that, we have used an in vitro model of oval cell lines expressing either a functional or non-functional c-Met receptor lacking tyrosine kinase activity, $Met^{flx/flx}$ and $Met^{-/-}$ oval cells, respectively. We demonstrate that HGF promotes a migratory and invasive response in oval cells that ultimately leads to cell rearrangement rather than to cell scatter. Additionally, we have explored the signaling mechanisms mediating HGF invasive response and have proved an essential

role for PI3K activation and metalloproteases (MMPs) expression and activation to achieve such response.

2. Material and methods

2.1. Reagents and antibodies

Mouse recombinant HGF was purchased from R&D Systems. ERK inhibitor UO126, p38 Inhibitor SB203580 and PI3K inhibitor LY294002 were from Calbiochem. The broad spectrum MMP inhibitor marimastat was kindly supplied by Dr. M. Quintanilla (IIB, Madrid, Spain). Dulbecco´s modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA were obtained from Gibco (Life Technologies). Type IV collagen and matrigel were from BD Bioscience. Penicillin, streptomycin, HEPES, bovine serum albumin (BSA), crystal violet dye, DNA oligos and buffer reagents were from Sigma-Aldrich. Oligo-dT was from Roche Diagnostics; RNeasy Kit from Quiagen; SuperScript III RNase H Reverse Transcriptase from Invitrogen (Life Technologies); ECL reagent and Glutathione Sepharose 4B beads (17-0756) were from GE Healthcare Europe. Vectashield mounting medium with DAPI was from Vector Laboratories. The anti-Occludin (711500) and anti-ZO-1 (617300) antibodies were from Invitrogen (Life Technologies). Anti-β-actin (clone AC-15) was from Sigma-Aldrich. Anti-PI3K p85 (06–195) was from Millipore; anti-GM130 (610823) from BD Bioscience and anti-Beta COP (NB120-2899) from Novus Biologicals. Anti-phosphotyrosine antibody (4G10, 05-321) and anti-Rac-1 (clone 23A8, 05-389) were from Millipore.

2.2. Cell lines and culture conditions

$Met^{flx/flx}$ and $Met^{-/-}$ oval cell lines were generated as described previously [14]. Early passage cells (passages 1 to 8) were maintained in DMEM supplemented with 10% FBS in a humidified incubator at 37ºC and a 5% CO_2 atmosphere. Medium was replaced every three days and cells passaged at 90% confluency. Prior to stimulation with growth factor cells were serum starved for 2-12 hours. Inhibitors were added 30 minutes to 1hour before addition of growth factor.

2.3. Cell scatter assays

Oval cells were allowed to grow as distinct individual colonies by seeding at low density (3400 cells/sq cm) in DMEM supplemented with 10% FBS. Next day, cultured cells were visualized under phase contrast microscopy and several fields of view were randomly selected and marked on the bottom of the plate using a paint marker to be able to follow up cell behaviour for the next 48 hours. Then, cells were stimulated with HGF (40 ng/ml) in DMEM-0% FBS and photographs of the selected fields were taken at different time points after treatment (0, 6, 16, 48h) under a phase contrast microscope using a 10X objective. Untreated cells seeded and maintained in the same culture conditions are used as control.

2.4. In vitro wound healing migration assays

Cells (34000 cells/sq cm) were seeded in Falcon 35mm-cell culture dishes and grown to confluence in complete medium. A sterile 10µl pipette tip was used to make a wound across a cell culture monolayer. Cell debris was removed by discarding the medium and washing three times with PBS. Cells were then incubated in DMEM-0% FBS supplemented or not with HGF (40 ng/ml). Multiple photographs of the wound were taken immediately after wounding (0 hours) and 48 hours later under a phase-contrast microscopy using NIS-Elements software (Nikon). The efficiency of the wound healing process was determined by calculating the area of the cell gap at the indicated times (0 and 48h), using ImageJ software. Three images were used for each wound at each experimental point and the experiment was always carried in triplicates. The results are expressed as percentage of healing at 48h with respect to zero time.

2.5. Invasion assays

In vitro invasion assays were performed using Transwell inserts (24-well plate inserts; 8µm pore size; BD Bioscience) coated either with reconstituted Matrigel basement membrane (0.3 mg/ml) or type IV collagen (7.5 µg/ml) according to the manufacturer's recommendation. 15000 cells were added to the upper chamber and incubated in the presence or absence of HGF (40 ng/ml) in DMEM-0% FBS. When kinase inhibitors were used, cells were serum starved for 2 hours and pretreated with the inhibitors for 1 hour prior to seeding on chambers. After 24 h in culture, cells in the upper chamber were carefully removed with a cotton swab and the cells that had invaded the membrane were fixed and stained with crystal violet 0.2% in 2% ethanol for 20 minutes. Several representative photographs of each well were taken. Quantification

of the invasion process was done by counting stained invaded cells in at least 20 fields/insert using phase-contrast microscopy (Eclipse TE300, Nikon) and a 10X objective. Each experiment was run in triplicates to confirm reproducibility of data.

2.6. Quantitative reversed transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA isolation, reverse transcription and quantitative PCR was performed as previously described [14,15]. Primer sequences used in the study are provided in supplementary information (supplementary Table 1).

2.7. Immunofluorescence analysis

To analyze protein expression by regular or confocal fluorescence microscopy, we used standard protocols previously described [14]. F-actin staining was performed using rhodamine-conjugated phalloidin following previous protocols [20]. Here we provide specific details on cell preparation, fixation and/or staining for particular antibodies. In all cases, cells were seeded on 2% gelatin-coated glass coverslips in DMEM supplemented with 10% FBS. For BetaCOP, GM130 and ZO-1 staining, cells were fixed with 4% paraformaldehyde for 20 min at RT followed by permeabilization in PBS containing 0.1% triton X-100-0.1% BSA for 20 min. After fixation-permeabilization, cells were incubated in blocking solution (2 %BSA in PBS, 1hour) and then incubated at 37ºC with primary antibodies (diluted 1:50 in PBS-1% BSA, 1 hour). Fluorescent-conjugated secondary antibodies (anti-mouse Alexa 488 or 594 and anti-rabbit Alexa 488) were diluted 1:200 in PBS-1% BSA and applied for 1h at RT. Cells were prepared for visualization by embedding in Vectashield mounting medium with DAPI and visualized in a Leica TCS-SL confocal microscope with a 63X objective or an Olympus BX-60 fluorescence microscope with a 60X objective.

2.8. Immunoblotting and immunoprecipitation

For total cell lysates preparation, Western blotting and immunoprecipitation assays we followed standard protocols described previously [14]. Specific details are as follows. Phosphorylated ZO-1 was detected by immunoprecipitation with an antibody against phosphotyrosine (4G10, 1 μg/ml) and anti-mouse IgG whole molecule-agarose antibody followed by Western blot analysis using an anti-ZO1 antibody (diluted 1:1000 in Tris-buffered saline containing 0.1% Tween 20 (TTBS) and 5% non-fat dried milk). For detection of occludin-1 (OCL-1), E-cadherin (E-CAD), β-actin (β-ACTIN) and p85

by Western blot the primary antibodies were diluted 1:500 to 1:2000 in 5% non-fat dried milk TTBS. Detection was done using horseradish-conjugated secondary antibodies (diluted 1:3000-1:5000 in 0.5% non-fat dried milk TTBS) and the enhanced chemiluminescence method.

2.9. Rac-1-GTP pull down assay

Cells were lysed in Magnesium Lysis Buffer (MLB) Pull Down Buffer (25mM Hepes (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1% NP40, 1% Glicerol, 10 µg/ml Leupeptine , 10 µg/ml Aprotinine, 1mM NaF and 1mM $NaVO_3$) and protein concentration was measured by Bradford assay. A GST-Pak-1 protein bound to glutathione sepharose beads was used to precipitate Rac-GTP from total cell extracts (0.5-1 mg of protein), essentially as described [21]. Rac-1 was detected by Western blotting and densitometric analysis was used to normalize Rac-GTP levels to total Rac-1 levels.

2.10. Small interfering RNA (siRNA) knockdown assays

Transient siRNA knockdown assays were performed as described previously [15]. PI3K regulatory subunit p85α siRNA SMART pool consists of four double-stranded siRNAs commercially designed and tested by Dharmacon (SMARTpool siRNA reagents; Dharmacon) whose sequences are: ACGCGAAGGCAACGAGAAA, UAAUAAACCACUACCGGAA, GAAGUCAAGCAUUGCGUCA, CUAAAAUGCACGGCGAUUA. siControl Non-targeting siRNA Pool (Dharmacon) was used as negative control. For siRNA transfection we used TransITsiQuest reagent (Mirus) according to the manufacturer´s recommendation and a final siRNA concentration of 50 nM. After transfection, cells were incubated for 24h in complete medium to allow efficient target down-regulation prior to protein harvesting or cell replating for invasion assays.

2.11. Statistical Analysis

Statistical analysis was performed by Students t-test method. The differences were assumed significant at $P < 0.05$.

3. Results

3.1.HGF promotes oval cell migration but not cell scattering.

Since HGF is known as Scatter Factor due to its ability to promote cell scatter on epithelial cells [17], we first run scatter assays with $Met^{flx/flx}$ oval cells, which have proved to respond to HGF [14]. Migratory cells were easily visualized both in untreated and HGF treated cultures, but interestingly, there was no cell scattering from the colonies. Instead, in the presence of HGF treatment cells clearly migrated to cluster rather than to scatter (Fig.1A). Furthermore, in a second stage of the migratory process, cell rearrangement was observed within the colonies in such a way that cells seemed to arrange in rows. This phenomenon was not seen in untreated cells. In these conditions, however, there was some cell loss due to cell sensitivity to serum deprivation, as previously described [14]. This migratory response elicited by HGF was not observed in $Met^{-/-}$ oval cells (data not shown). To further test the effect of HGF on oval cell migration we also run in vitro wound healing assays. As seen in Fig. 1B-C, oval cells intrinsically attempted to migrate toward the wound but this capacity was clearly increased in the presence of the growth factor, effect that was not seen in $Met^{-/-}$ oval cells, proving its specificity. Furthermore, consistently with our initial observations, wound directed oval cell migration appeared to show a collective rather than a single cell migration mode. Altogether, our data demonstrates that HGF promotes oval cell migration. However, it does not act as a scatter factor.

3.2. HGF-triggered migratory response in oval cells is associated with reorganization of cytoskeleton and cell-cell contacts but not an EMT process.

Cell migration implies changes in F-actin cytoskeleton [22]. Therefore, using standard culture conditions that allow free cell movement, that is, low cell densities similar to those used for scatter assays, we analyzed by confocal microscopy the cytoskeletal F-actin pattern in oval cells after treatment with HGF. HGF-treated mouse oval cells displayed a highly dynamic and dense actin network. Thus, F-actin-enriched structures including lamelipodia and filopodia were seen at the migratory front of individual migrating cells and cells at the edges of the colonies (Fig. 1D), indicating a continuous reorganization of actin cytoskeleton. Accordingly, HGF activated Rac1 (Fig. 1E), one of the key signaling components controlling actin cytoskeleton organization and cell migration [23]. It is worth mentioning that all experiments were done in the absence of a matrix substrate. However, there is a critical physical and functional link between actin cytoskeleton and ECM. Therefore, we analyzed whether HGF might

induce the expression of ECM proteins and integrin receptors in oval cells. Indeed, after 24 hours of treatment with HGF we observe an induction in the expression of integrin alpha2 and beta2, collagen 1a1, laminin3 and fibronectin1 mRNAs, but no regulatory effect was seen on collagen 3a1, proving the specificity of the response (Supplementary Figure 1).

In parallel, we looked for changes in expression and/or subcellular localization of cell-to-cell contact proteins. We did not detect any obvious differences in mRNA or protein levels of E-cadherin, a member of the cadherin protein family that forms part of the adherens junctions (Fig. 2A,B). Similarly, ZO-1 and OCCLUDIN-1, two protein components of tight junctions (TJ), were unaltered by HGF treatment (Fig. 2B). Nevertheless, we analyzed the subcellular localization pattern of E-cadherin by confocal microscopy to see whether HGF provoked any alteration in the E-cadherin distribution but we could not identify clear differences in localization (supplementary Fig.2A). When we analyzed ZO-1 localization pattern we observed a switch from cell-to-cell contact sites to a more diffuse intracellular staining pattern (Fig. 2C). Furthermore, in agreement with previously published data [24], ZO-1 cytoplasmic relocalization was correlated with activation of the protein by phosphorylation, as evidenced by immunoprecipitation assays (Fig. 2D). These results suggest that HGF induces profound changes in cell-to-cell contacts in oval cells. Importantly, all the described events were abolished in $Met^{-/-}$ oval cells, which demonstrate that they depend on the tyrosine kinase activity of HGF receptor, c-Met.

Induction of a migratory phenotype is often associated with cell phenotypic change, more specifically, with an epithelial-mesenchymal transition (EMT). This has been also described in HGF-triggered migration on liver cancer cell lines [25]. Here, we describe that the pro-migratory activity triggered by HGF in oval cells is accompanied by changes in F-actin cytoskeleton and subcellular localization of ZO-1. However, our results do not evidence a switch to a mesenchymal phenotype nor down-regulation of E-cadherin or cell dissociation, all of them being typical characteristics of EMT. These observations seemed to discard a potential induction of an EMT process by HGF in the oval cell model. Nevertheless, to further demonstrate our hypothesis we measured Snail mRNA levels, the best known master regulator of EMT [26]. We could not detect any induction of Snail mRNA expression upon treatment with HGF (Fig. 2E). It is worth noting that HGF failed to induce Snail or down-regulate E-cadherin expression

regardless of the absence or presence of matrix as culture substrate (supplementary Fig.2B). We run parallel analysis using wound healing assays, as a culture condition to push the migratory phenotype of the cells. Results evidence that wound healing by itself provokes an induction of Snail and moderate down-regulation of E-cadherin in a time-dependent manner (supplementary Fig. 3A). However, HGF treatment did not further induce Snail and interestingly, prevents E-cadherin down-regulation. Consistently, HGF did not induce loss of expression of cell junctions proteins or changes in E-cadherin staining pattern (supplementary Fig. 3B-C). As expected, F-actin cytoskeleton pattern is consistent with a migratory cell phenotype both in untreated and HGF-treated, but aditionally, under HGF treatment F-actin fibers aligned along the cells rows are seen, supporting an oriented cell movement toward the wound (supplementary Fig. 3C). All these results demonstrate that even under wound healing conditions HGF-triggered pro-migratory activity is not associated with EMT or loss of cell-to-cell contact proteins.

3.3. HGF-triggered migratory response in oval cells is associated with changes in Golgi organization and polarity.

Apart from the absence of cell scattering, one of the most interesting observations was the cell rearrangement induced by HGF within the oval cell colonies. This prompted us to hypothesize on a non-random oriented/polarized migratory response. In an attempt to study such hypothesis we analyzed the Golgi complex (GC) distribution since it has been described that polarized migratory cells display the GC oriented toward the direction of migration and this frontwards polarization has been proposed to be involved in directional migration [27]. For that, we stained GC using an antibody against B-COP, a coat protein of nonclathrin-coated vesicles of the GC, or an antibody against GM130, a cis-Golgi matrix protein, and analyzed it by confocal microscopy. As seen in Fig. 3A, HGF-treated $Met^{flx/flx}$, but not $Met^{-/-}$, oval cells presented a tightly packed Golgi structure as evidenced by B-COP staining. This effect was more prominent after long-term treatment, coinciding with cell rearrangement. Quantitative analysis demonstrated that HGF significantly increased the percentage of cells showing high Golgi compaction (GC occupies less than $1/4^{th}$ of cell surface), whereas decreased the number of cells with medium (between $1/4^{th}$ and $1/2^{th}$ of cell surface) or none (more than $1/2^{th}$ of cell surface) Golgi compaction (Fig. 3B). Identical results were obtained when using anti-GM130 antibody (supplementary Fig. 4). A detailed observation of Golgi position let us conclude that while in leading cells GC

appears to face direction of migration, cells on rearer positions show a heterogeneous pattern, not being clear a perfect correlation with the direction of cell migration. To further clarify this issue, we performed Golgi staining in wound healing assays, a best characterized model for directed cell movement. Image in Fig. 3C shows a migrating front towards wound edge in HGF-treated cells. As seen in standard cultures, GC compaction was very clear under these conditions. The GC of the cell at the front of the group was facing the wound whereas the rest of the cells showed a more heterogeneous positioning pattern with predominance of lateral position. All these results further demonstrate a HGF-induced migratory response in oval cells and at the same time support a collective migration pattern.

3.4. HGF increases oval cell invasion through extracellular matrix: involvement of matrix metalloproteinases (MMPs).

Once we had demonstrated that the HGF/Met pathway induces a complex and specific migratory response in oval cells, we questioned whether this signaling pathway could also regulate oval cell invasion capacity through extracellular matrix substrates. With this aim, we performed additional experiments using transwells coated either with collagen IV or matrigel. Results demonstrated a significant increase in the number of cells that migrated and invaded to the bottom side of the filters upon HGF treatment as evidenced by the microcopy images and quantitative analysis (Fig. 4A-C). Once again, no significant differences were observed between untreated and treated $Met^{-/-}$ oval cells. MMPs are major proteolytic enzymes known to play a central role during extracellular matrix degradation and remodeling, therefore being key players in cell invasion [28]. Their expression and activity can be modulated by growth factors and cytokines. In fact, HGF has been reported to increase the expression of several members of the MMP family to mediate tubulogenesis or to increase tumor cell invasiveness [29-31]. This prompted us to analyze the expression of MMPs, including MMP2, 3, 7, 9, 10, 11, 13, 14, as well as TIMP1 and 3, two members of the tissue inhibitors of metalloproteinases (TIMPs), key regulators of MMPs [32]. As shown in Fig. 4D, we found up-regulation of MMP3, 9, 10 and 13 mRNAs in oval cells treated with HGF, both in normal cultures (on uncoated plastic dishes) or in cells cultured on matrigel-coated dishes (data not shown). No significant changes were found in the other MMPs analyzed (data not shown) or in TIMP 1 and 3 (Fig. 4D). To test whether the MMP matrix degrading activity was important for oval cell invasion we run invasion assays in the same

conditions to those performed before but in the presence of marimastat, a well-known broad spectrum MMP inhibitor. Treatment of cells with this inhibitor partly but significantly decreased basal and HGF-stimulated cell invasion (Fig. 4E), strongly suggesting their contribution to cell invasion.

3.5. PI3K activation is critical for the HGF-induced oval cell migratory/invasive response in oval cells.

Next, we aimed at clarifying the HGF-triggered signaling pathways mediating the migratory/invasive response elicited by this growth factor in oval cells. Before, we have reported that HGF through c-Met activates PI3K/AKT and ERK1/2 MAPKs pathways [14] in oval cells. We have also demonstrated HGF/c-Met-mediated activation of p38MAPK in these cells (supplementary Fig. 5). Furthermore, HGF/c-Met-triggered signaling is not significantly altered when oval cells are cultured on collagen IV or matrigel (supplementary Fig. 5). To analyze the role of these signaling pathways in the HGF pro-invasive activity we used selective inhibitors, including LY294002 (PI3K inhibitor), UO126 (ERK1/2 MAPK inhibitor) and SB203580 (p38α/β MAPK inhibitor). Treatment of cells with LY294002 completely abolished HGF-induced invasive response, both on collagen IV and matrigel (Fig. 5A, B). SB203580 inhibitor only blocked HGF effect on collagen IV but not matrigel, evidencing a potentially differential role for this signaling pathway depending upon the extracellular matrix substrate used. Regarding UO126, no significant effect was observed on cell invasion either through matrigel or collagen IV. In summary, these results pointed to a critical role for PI3K during HGF-induced oval cell migration/invasion. To confirm such a role, we carried out siRNA-mediated transient silencing experiments using siRNAs specific for p85, the regulatory subunit of PI3K. We reached an average gene-silencing efficiency of 70-80%. A western blot of a representative silencing experiment is shown (Fig. 5C). As seen in Fig. 5D and Fig. 5E, p85 knockdown blocked HGF-induced oval cell invasion, thus demonstrating that activation of PI3K is required for this effect.

Our data evidenced that MMPs contribute to HGF-induced oval cell invasion. The signaling mechanisms mediating MMPs regulation by HGF remain elusive. Therefore, we ought to analyze whether they are direct targets for p38 α/β MAPK and/or PI3K, the two signaling kinases playing a role in HGF pro-invasive activities in these cells. Indeed, up-regulation of MMP 9, 10 and 13 mRNAs was partly or totally

inhibited in the presence of p38MAPK and PI3K inhibitors. MMP3 mRNA expression was not regulated by these inhibitors suggesting a differential regulation by other signaling pathways. Additionally, p85 silencing prevented the HGF-mediated up-regulation of MMP 9 and 10 mRNAs expression. Strikingly, MMP13 induction, which was inhibited by LY294002, was not significantly affected by p85 silencing (Figure 5F and 5G). Therefore, all these results prove for the first time that HGF promotes expression of some MMPs in oval cells via PI3K and p38MAPK activation. Hence, HGF acting through PI3K and p38MAPK regulates MMPs expression, which would allow cell invasion.

4. Discussion

Studying the behaviour of oval cells in response to regulatory factors appears more than ever fundamental to fully understand their relevance in liver pathophysiology , their precise role during regeneration after severe injury and whether or not they are potentially manipulable to further stimulate regenerative responses safely. Here, we demonstrate that HGF through c-Met promotes a complex migratory and invasive response in oval cells, which we propose it is part of a morphogenic program activated in these cells during their expansion in the damaged liver. We also uncover some of the major c-Met-dependent mechanisms driving such response.

The motogenic activity induced by HGF in epithelial cells has been extensively studied and has led to a classical model in which HGF acts as a scatter factor in standard bidimensional culture, whereas in tridimensional culture on collagen it promotes the formation of long and branched tubular structures, where cell-cell junctions are maintained [33]. Tubulogenesis is believed to reflect the morphogenic activity promoted by the HGF/c-Met pathway that in the case of hepatic cells is displayed as the formation of cord-like structures that seem to emulate the normal hepatic plate architecture [34,35]. Different from this classical response, we do not see scattering in oval cells in response to HGF. Furthermore, the row-like organized morphology of the oval cell culture in the presence of HGF could at least partially recapitulate the migratory process of oval cells into the parenchyma during their expansion in the damaged liver. However, such structures are observed in the absence of a matrix substrate, showing a higher plasticity of oval cells for self-organization into epithelial structures, which is most likely associated with their progenitor nature. It is also plausible to hypothesize that

induction of the expression of ECM proteins and integrin receptors may well contribute to the HGF-triggered migratory response observed in these cells.

Additionally, our data demonstrate that HGF-promoted migratory/invasive response in oval cells is not associated with EMT. The role of EMT in the pro-migratory/invasive activities of HGF are not at all clear. It has been postulated as a key mechanism to allow cells move through extracellular matrix during the early stages of the invasive growth program induced by HGF in MDCK cells. However, this EMT process is at most transient and partial since the cells ultimately remain attached to each other [36]. Differently, the scatter response induced in liver tumor cells depends on Snail induction mediated by MAPK activation [25]. Likewise, a positive feedback loop via HGF contributes to maintain a mesenchymal phenotype in HCC cells, in turn, favoring invasion and tumor progression [37]. These observations evidence the capacity of HGF to induce total and partial EMT but such activities depend very much on cell type and context. On the other hand, in spite of the lack of effect on ZO-1 expression and the absence of cell dissociation, our data demonstrate activation and cytosolic translocation of this protein. Strikingly, this has been associated with stimulation of cell dissociation and migration [24]. However, it has also been demonstrated that changes in ZO-1 localization during HGF-induced morphogenesis of polarized MDCK cells do not imply loss of functional integrity of TJ [38]. Besides, the specific pattern of Golgi observed during HGF-induced migration in oval cells is quite similar to that described during HGF-induced in vitro morphogenesis of MDCK cells [39]. Putting it all together, we believe our observations are consistent with the induction of a collective-type migration as a key component in the specific morphogenic response elicited by HGF in oval cells. It is worth to mention that parallel experiments that are being performed in our laboratory using a wt mouse immortalized neonatal hepatocyte cell line as a model of differentiated liver epithelial cells have shown a differential pro-migratory and invasive response to HGF. Although HGF also promotes migration and invasion through ECM not associated with cell scattering or EMT, neither ZO-1 translocation nor Golgi compaction or cell rearrangement are seen (Supplementary Figure 6). These results evidence that in spite of many similarities the morphogenic responses to HGF might indeed differ in hepatic cells at different stages of differentiation. Nevertheless, further investigation is needed to clarify the cellular and molecular mechanisms behind these differential responses.

Collective migration has been proved to be important during the morphogenic responses ocurring in tissue development and regeneration but it can also participate in invasive and metastatic processes in tumors [40]. This type of migration can provide clear advantages for the regenerative response, such as higher production of pro-regenerative autocrine factors and proteases to facilitate ECM degradation, or protection of cells against the attack of inflammatory cells infiltrated in the damaged tissue. Regarding ECM degradation, we demonstrate that HGF induces the expression of several metalloproteases in oval cells. MMPs expression and activation contribute to HGF-mediated scattering, wound-healing and invasion in normal and tumoral hepatic cells [29-31]. More importantly, MMPs-mediated ECM remodelling are fundamental processes that control collective cell migration and invasion during tissue patterning [41], an example of that being tissue remodeling during liver regeneration [42]. Consistent with this and the crucial pro-regenerative role of HGF, lack of c-Met results in aberrant tissue remodelling during stem cell-mediated liver regeneration, associated with a reduction in MMP9 activity and oval cell migration into parenchyma [16]. Our data indicate that MMPs contribute to both basal and HGF-stimulated oval cell migration/invasion through ECM. Nonetheless, we have to keep in mind that MMPs do much more than degrade physical barriers. They are also critical for growth factors activation. Thus, MMP-13 activates HGF by converting the precursor form into the mature form, which has been proved to accelerate recovery from experimental liver fibrosis [43], so future studies will allow us to clarify the net contribution of MMPs in the oval cell response to HGF.

The analysis of the signalling mechanisms involved in the pro-migratory/invasive response of HGF in oval cells reveals a crucial role for PI3K. Given the multifunctional nature of HGF/c-Met signalling pathway, there has been from the beginning an enormous interest in elucidating the signals mediating the different biological activities triggered by HGF/c-Met. Strikingly, PI3K activation, shown to mediate essential antiapoptotic signal [15,44] has also been reported to be critical for cell dissociation [45]. Interestingly, cooperation between multiple signaling pathways is needed to induce HGF morphogenic effects. p38MAPK cooperate with ERKs to promote HGF-mediated cornea epithelial cell migration [46]. Furthermore, concomitant activation of Ras/ERKs, PI3K/AKT and Rac1/p38 is necessary to reach maximal migratory capacity in cortical neurons treated with HGF [47]. Why p38 appears to only

be a major participant in oval cell migration/invasion through collagen IV is not obvious. We could hypothesize that it might depend on the MMPs regulated by p38, able to degrade collagen but not other ECM proteins in oval cells. Indeed, p38 has been involved in MMP1 induction and collagen degradation by HGF [48]. However, p38 targets not only collagenases but different types of MMPs in oval cells. Certainly, the substrate is important to modulate cellular response. For instance, HGF scattering effect on MDCK is more efficient on collagen I and fibronectin than on laminin I [49], which is associated to differences in F-actin cytoskeleton organization and formation and turnover of integrin-mediated focal adhesions. p38 also controls F-actin cytoskeleton rearrangement during PDGF-induced migration [50]. Clearly, several interesting research avenues remain open to clarify the precise role played by specific signals in the HGF pro-migratory/invasive response in oval cells. Nonetheless, we provide novel mechanistic insights on how HGF regulates MMPs in oval cells and demonstrate that HGF induces the expression of specific MMPs (MMP9, 10, 13) acting through PI3K and p38MAPK. These and other signaling pathways have been involved in regulation of several MMPs by different ligands in other cell types, evidencing a complex, context and stimulus-dependent regulation [51-54]. This would explain why we do not see regulation of MMP3 by our targeted kinases. We do not have a straightforward explanation for the discrepancy between the effect of PI3K inhibitor and p85 silencing on MMP13 expression. However, a PI3K activity-independent action of p85 regulatory subunit might be possible, as previously described in other contexts [55]. Moreover, PI3K can be also activated in a p85-independent manner [56]. Further investigation is needed to clarify this issue, but overall we provide the first characterization of the signaling cascades involved in the regulation of MMPs by HGF in oval cells.

Importantly, the fact that PI3K inhibition completely abolishes HGF-induced invasive response strongly supports a central role for this pathway and suggests a functional dependency between PI3K and p38 for collagen invasion, further supported by the fact that PI3K and p38 share some MMPs as targets. Given that p38 can be a downstream effector of Rac1 [57] and Rac1 an effector of PI3K [47] our data would support a signaling axis HGF/c-Met/PI3K/Rac1/p38/MMPs as a major mechanism mediating the migratory/invasive response of oval cells through collagen.

In conclusion, we not only demonstrate the pro-migratory/invasive activity of HGF on oval cells, but provide solid data on the cellular and molecular mechanisms

mediating such activity. The pro-regenerative effect of the HGF/c-Met signaling pathway is unquestionable. The HGF/c-Met-dependent migratory/invasive response described here, together with its antiapoptotic activity [14] constitute critical components in any morphogenic and regenerative response. Therefore, we believe that our in vitro observations recapitulate the epithelial morphogenic program induced by HGF during the liver regenerative response involving oval cells. We cannot rule out the possibility that the collective pro-migratory/invasive response promoted by HGF/c-Met in oval cells might contribute to the pro-fibrotic/pro-tumoral function of these cells upon disruption of the maturation process and subsequent malignant conversion. However, this hypothesis becomes harder to sustain if we take into account the over-demonstrated antifibrogenic and therapeutic action of HGF/c-Met signaling in liver and the severe detrimental effects seen upon c-Met deletion [58,59]. Future studies will help us to clarify these issues, which can provide a step forward for the development of new therapeutic approaches to fight liver chronic diseases.

Figure legends

Figure 1. Effect of HGF on oval cell migration, F-actin cytoskeleton and Rac1 activity. A. Scatter assays. $Met^{flx/flx}$ oval cells were cultured at low density on plastic dishes with or without HGF treatment (40 ng/ml). Representative phase contrast images of a randomly selected area of the dish are shown. Scale bar=100 μm. **B.** Wound healing assays. $Met^{flx/flx}$ and $Met^{-/-}$ oval cells were cultured at confluency on plastic dishes. Next day a wound was done using a sterile tip and cells were cultured during 48h with or without HGF (40 ng/ml). Phase contrast images were taken at zero time (after wounding) and 48h later. Scale bar=100 μm. **C.** Quantitative analysis of wound closure after 48h. Data are expressed as % of closure and are the mean±SEM of four independent experiments. ** P<0,01 HGF-treated vs untreated $Met^{flx/flx}$. **D.** Confocal microscopy images of F-actin staining in $Met^{flx/flx}$ oval cells untreated or treated with HGF (40 ng/ml) for 16 or 24h. Illustrative lamelipodia and filopodia structures are indicated by arrowheads and arrows, respectively. Representative images out of 4 experiments are shown. Scale bar= 45μm. **E.** Analysis of Rac-1 activation by pull-down assays in $Met^{flx/flx}$ oval cells untreated or treated with HGF (40 ng/ml) for 5 and 10 min. A representative experiment out of 5 is shown.

Figure 2. Effect of HGF on cell-cell adhesion proteins expression, ZO-1 subcellular localization and activation. A. qRT-PCR analysis for E-cadherin gene expression in $Met^{flx/flx}$ and $Met^{-/-}$ oval cells cultured for 24 and 48h with or without HGF (40 ng/ml). Data represent RQ values and are mean±SEM of 6 experiments. **B.** Western blot analysis for ZO-1, Occludin-1 (OCL-1) and E-cadherin (E-CAD) protein levels. $Met^{flx/flx}$ and $Met^{-/-}$ oval cells were cultured for 24 and 72h with or without HGF (40 ng/ml). Total protein lysates were collected and used for Western blot analysis. β-actin was used as loading control. **C.** Confocal microscopy images of ZO-1 staining in $Met^{flx/flx}$ and $Met^{-/-}$ oval cells either untreated or treated with HGF (40ng/ml) for 24h. Representative images out of 4 experiments are shown. Scale bar= 20 μm. **D.** Analysis of ZO-1 activation by immunoprecipitation with anti-phosphotyrosine antibody followed by immunodetection of ZO-1 with a specific antibody, in cells cultured for 24h with or without HGF (40 ng/ml). Total ZO-1 protein levels in the same lysates analyzed by western blot are shown as loading control. A representative experiment out of 4 is shown. **E.** qRT-PCR analysis for Snail gene expression in $Met^{flx/flx}$ and $Met^{-/-}$ oval cells cultured for 24 and 48h with or without HGF (40 ng/ml). Data represent RQ values and are mean±SEM of 6 experiments.

Figure 3. Effect of HGF on GC subcellular distribution in oval cells. A. Cells were cultured untreated (Control) or treated with HGF (40ng/ml) for the indicated time periods. Then, they were fixed and stained with B-COP antibody to visualize GC by confocal microscopy. Representative images out of 3 experiments are shown. Scale bar=50 μm. **B.** Quantitative analysis of GC compaction degree. Cells were divided in three categories depending upon the degree of compaction: High (GC extended around $1/4^{th}$ of nucleus surface or less), Medium (GC extended between $1/4^{th}$ and 1/2th of nucleus surface), None (GC extended more than 1/2th of nucleus surface). Data are expressed as % of cells in each category and are mean±SEM of 3 experiments. **C.** Confocal microscopy images of ZO-1 (green fluorescence) and GM130 (red fluorescence) staining in $Met^{flx/flx}$ cells at 48h after wounding and treatment with HGF (40 ng/ml). Representative images out of 2 experiments are shown. Scale bar=50 μm.

Figure 4. Analysis of the effect of HGF on oval cell invasion through extracellular matrix. Role of MMPs. A-C: Cells were seeded on the upper chamber of transwells coated with collagen IV or matrigel and incubated for 24h in the absence or presence of

HGF (40 ng/ml). Cells that had invaded the bottom chamber were fixed and stained with crystal violet and analyzed by Microscopy. **A.** Representative phase contrast images of the different culture conditions are shown. Scale bar= 100 μm. **B and C.** Quantitative analysis of cell invasion. Data are expressed as number of invaded cells and are mean±SEM of at least 5 experiments performed in duplicates. ** P<0.01 and ***P<0.001 respect to untreated cells. **D.** qRT-PCR analysis for MMP3, 9, 10, 13, TIMPs 1 and 3. $Met^{flx/flx}$ cells were seeded on uncoated plastic dishes and cultured untreated or treated with HGF (40ng/ml) for 24h. Total RNA was isolated and used for qRT-PCR analysis. Data represent RQ values and are mean±SEM of at least 4 experiments. * P<0.05; **P<0.01 respect to untreated. **E.** Effect of MMP inhibition on oval cell invasion. Invasion assays in the absence or presence of HGF (40 ng/ml) and/or marimastat (10 μM) were conducted. Data represent number of invaded cells expressed as fold change with respect to untreated cells and are mean±SEM of 2 independent experiments run in duplicate-triplicate. ***P<0.001.

Figure 5. Analysis of the signaling pathways mediating HGF-induced oval cell invasion. A,B: Quantitative analysis of cell invasion through collagen IV (**A**) or matrigel (**B**) in $Met^{flx/flx}$ oval cells treated in the absence or presence of HGF (40 ng/ml), LY294002 (5 μM), UO126 (5 μM) or SB203580 (10 μM). Data are expressed as fold change with respect to untreated cells and are mean±SEM of at least 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001. **C.** Western blot analysis of the p85 silencing efficiency. $Met^{flx/flx}$ cells were transfected with p85 specific siRNAs (50 nM). Next day, cells were treated (H) or not (C) with HGF (40 ng/ml) for 24h. Total lysates were collected and used for Western blot detection of p85 using a specific antibody. β-actin was used as loading control. The ratios of p85 to β-actin obtained by densitometric analysis are shown and expressed as fold change with respect to control (untreated cells transfected with non-targeting siRNAs, NT). **D,E**: Effect of p85 silencing on HGF-induced oval cell invasion. 24h after transfection with non-targeting siRNAs (NT) or p85 specific siRNAs (si p85) cells were trypsinized and seeded on transwells chambers coated with collagen IV **(D)** or matrigel **(E)** and cultured for 24h with or without HGF (40 ng/ml). Data are expressed as fold change with respect to control and are mean±SEM of 2 (collagen) or 5 (matrigel) independent experiments run in duplicate-triplicate. *P<0.05; **P<0.01. **F**: Effect of kinase inhibitors on HGF-induced MMPs expression. $Met^{flx/flx}$ cells were seeded on uncoated plastic dishes and cultured untreated

or treated with HGF (40ng/ml), LY294002 (5 μM) or SB203580 (10 μM) for 24h. Total RNA was isolated and used for qRT-PCR analysis. Data represent RQ values and are mean±SEM of 2-3 experiments. * P<0.05; **P<0.01 respect to untreated. **G**: Effect of p85 silencing on HGF-induced MMPs expression. 24h after transfection with non-targeting siRNAs (NT) or p85 specific siRNAs (si p85) cells were serum-starved and treated or not with HGF (40 ng/ml) for 24h. Total RNA was isolated and used for qRT-PCR analysis. Data represent RQ values and are mean±SEM of 2 experiments. P<0.05; **P<0.01; ***P<0.001.

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Figure 1 (double column fitting figure)

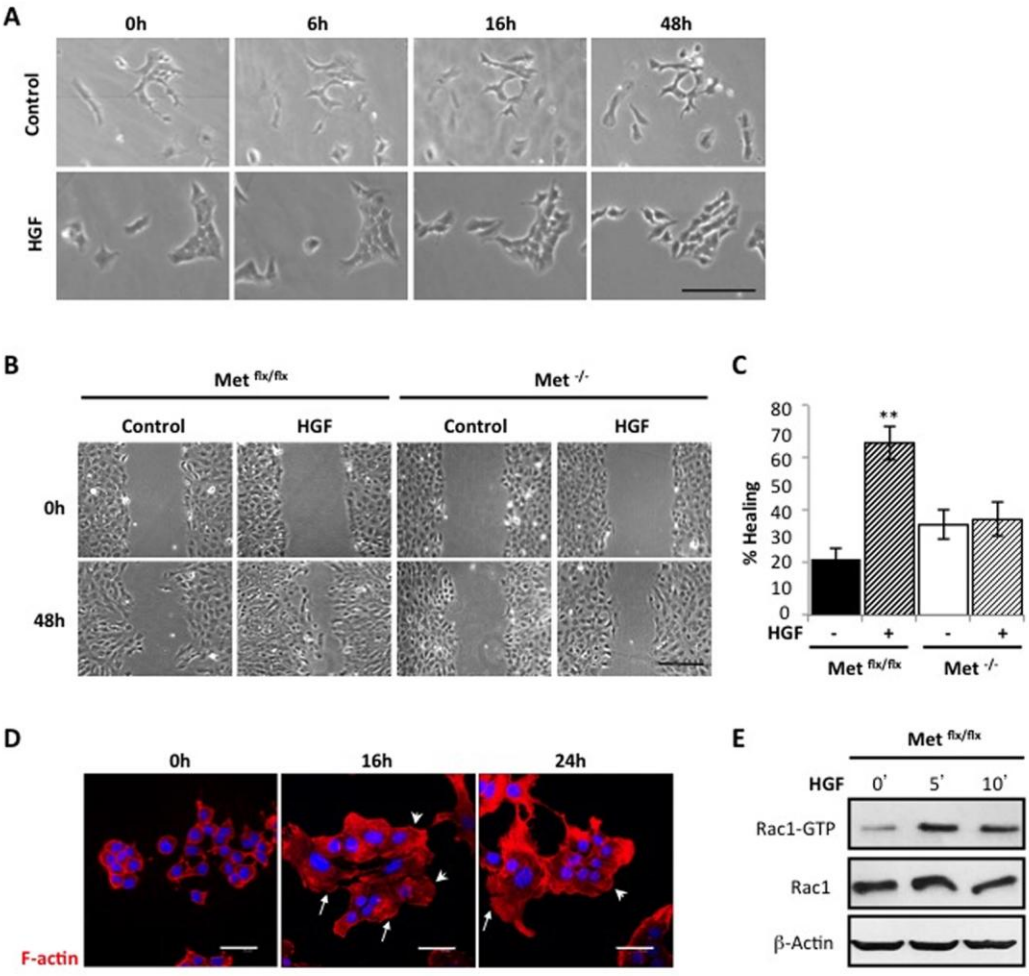


Figure 2 (double column fitting figure)

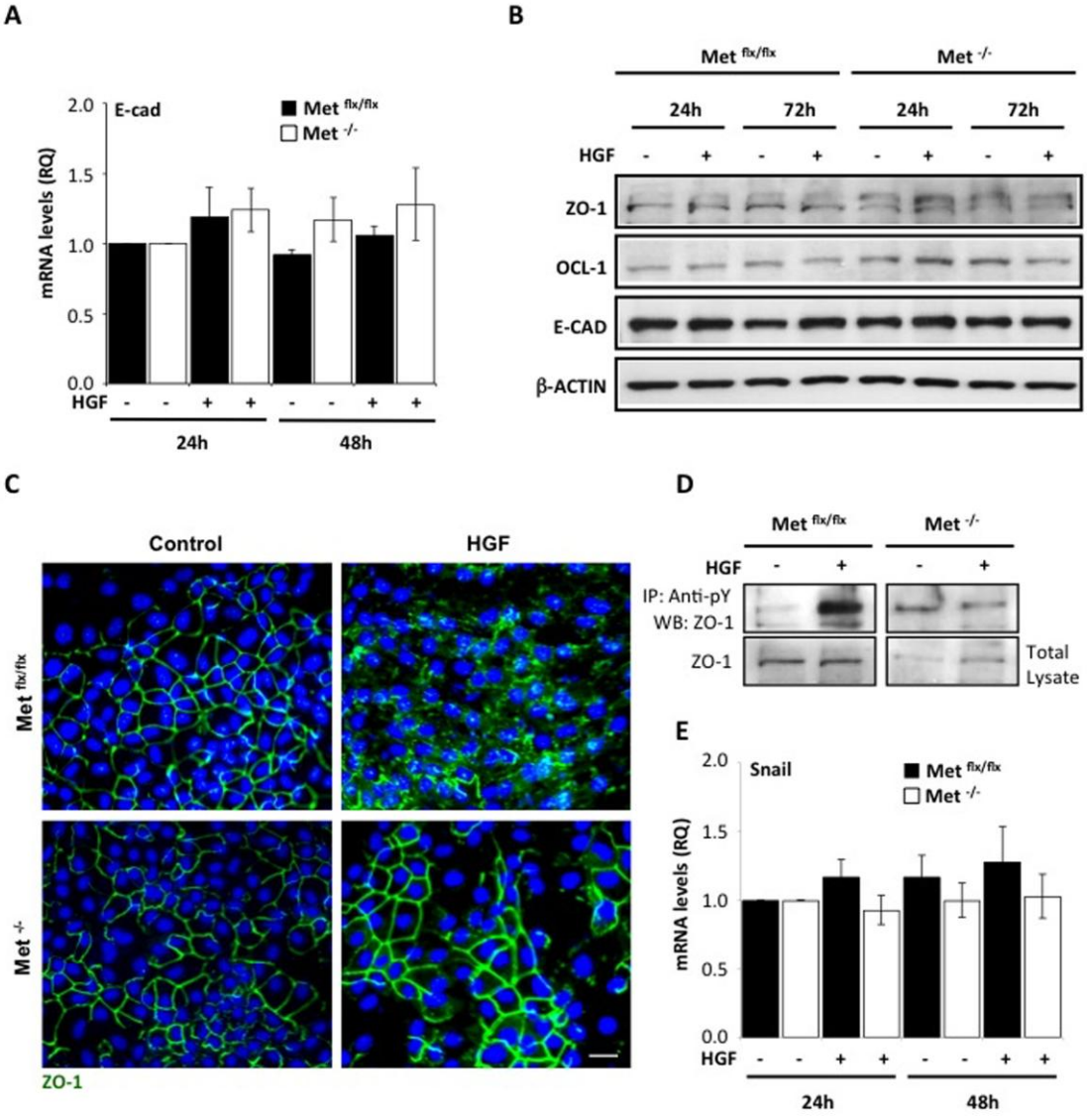
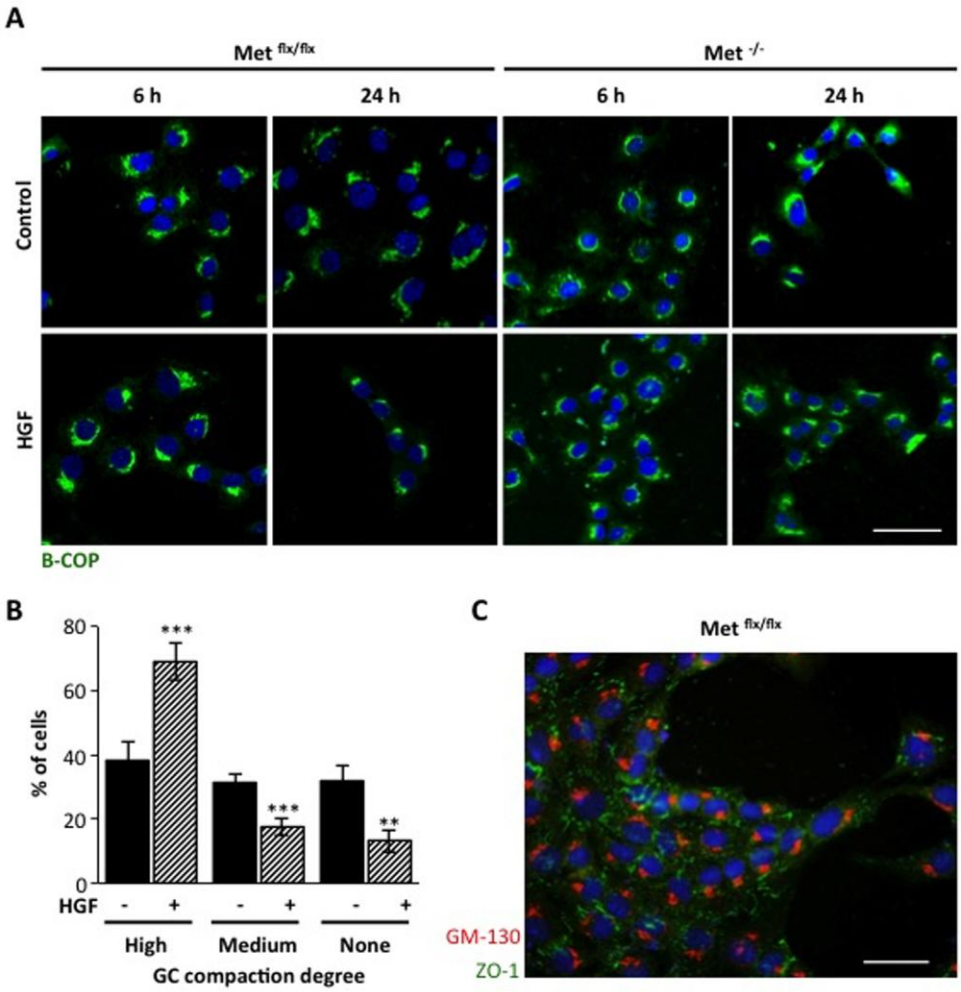


Figure 3 (1.5 column fitting figure)



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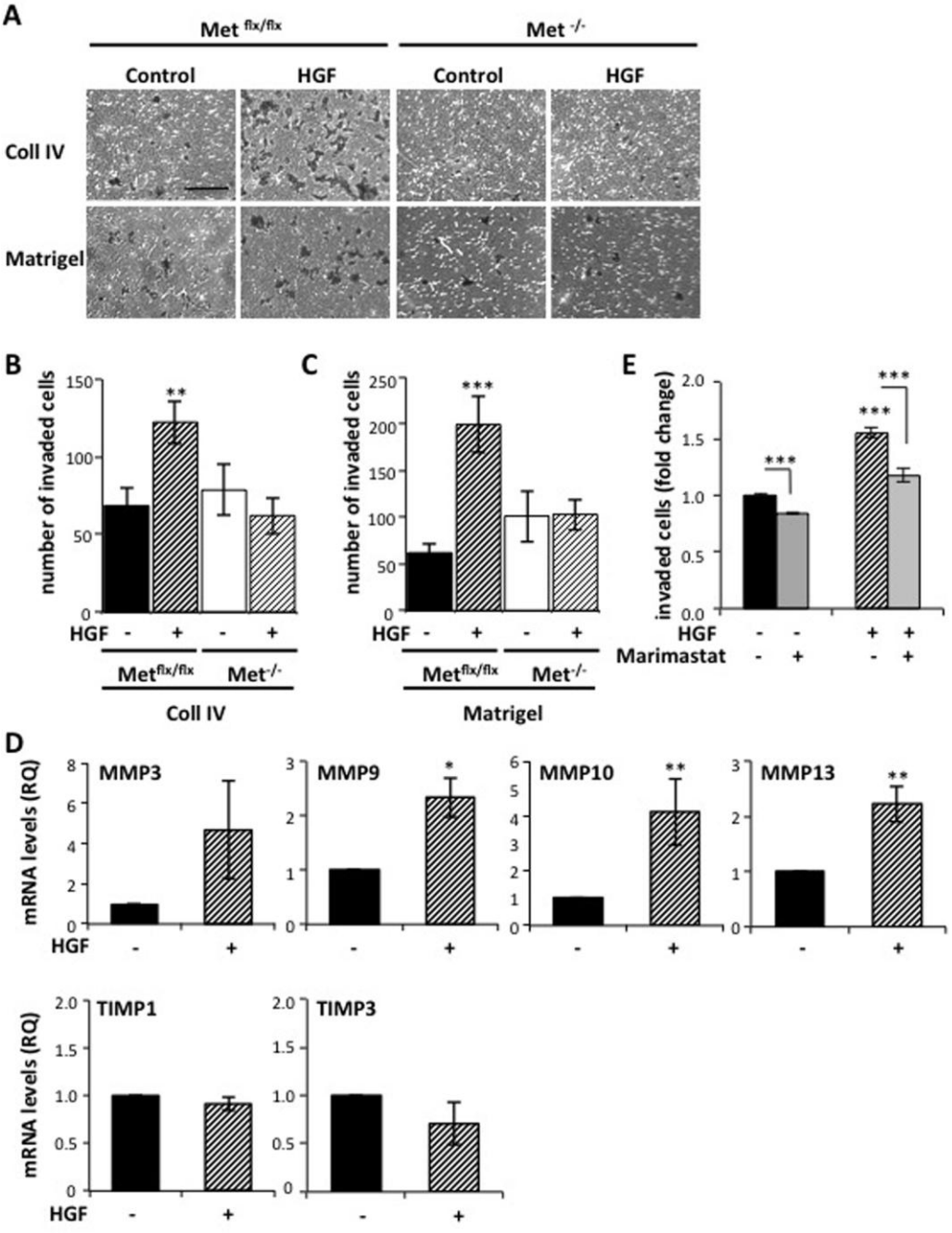

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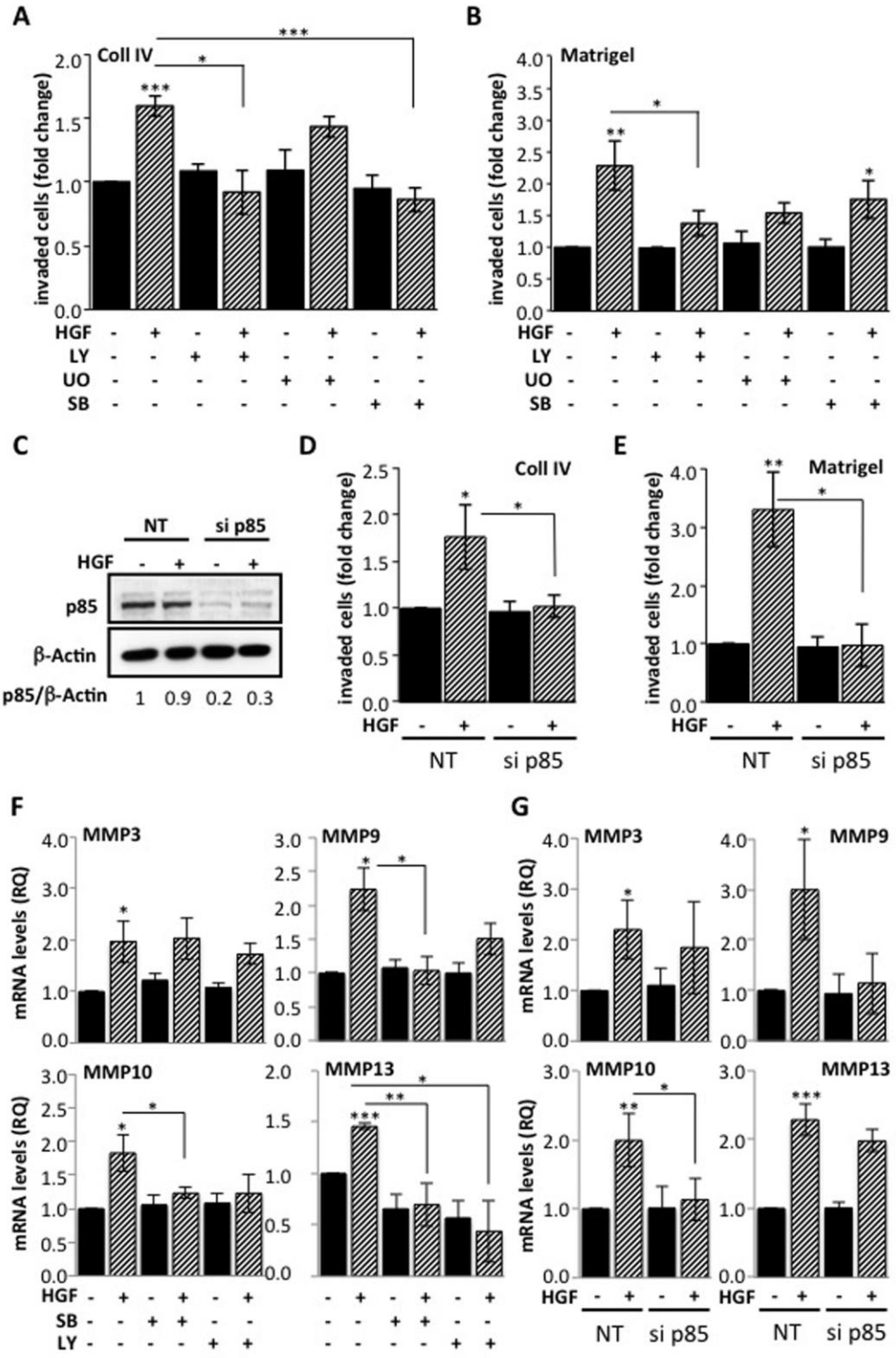

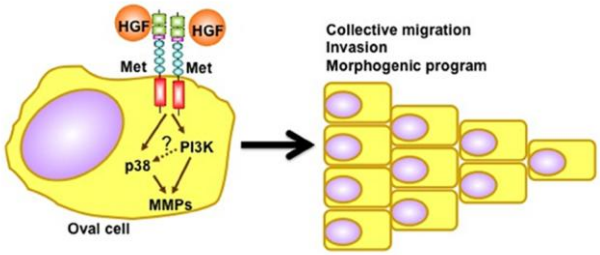

Graphical abstract

Highlights

- HGF induces c-Met-dependent oval cell morphogenic collective migration.
- HGF-induced migratory response does not involve cell scattering or EMT.
- HGF induces c-Met dependent oval cell invasion through ECM.
- PI3K is critical for the HGF/c-Met-dependent oval cell migratory/invasive response.